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ANALYSIS OF GENETIC CHARACTERISTICS

OF INFLUENZA VIRUS A/CHICKEN/CHELYABINSK/30/2019 H9N2 ISOLATED IN CHELYABINSK OBLAST

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SUMMARY

The paper presents data on the study of genetic characteristics of the influenza virus A/chicken/Chelyabinsk/30/2019 H9N2 isolated from pathological material (chicken internal organs) in February 2019 and received from the poultry farm in the Chelyabinsk Oblast. The H9N2 subtype of the isolated virus was identified based on virological analysis. Sequencing of the hemagglutinin gene segment revealed that the amino acid sequence at the cleavage site was RSSR/GLF, which is characteristic of a low virulent avian influenza virus. Phylogenetic analysis of the obtained nucleotide sequences of the hemagglutinin gene fragment (1–1,539 bp open reading frame) showed that the A/chicken/Chelyabinsk/30/2019 H9N2 isolate belongs to the G1 genetic group of the low virulent influenza virus A/H9, the representatives of which are widely spread in the Middle Eastern and Central Asian countries. The complete nucleotide genome sequence of the studied pathogen was determined. The comparative analysis of all genomic segments using the GenBank database revealed a close relationship (over 99%) between the A/chicken/Chelyabinsk/30/2019 H9N2 virus and the A/H9 influenza virus isolates circulating in Israel in 2006–2012. According to the analysis of the predicted amino acid sequence of the studied isolate, the positions of some molecular markers that determine the biological properties of the virus have been identified. Most amino acid positions of hemagglutinin (according to H3 subtype sequence numbering) suggest affinity for the ACA2-3Gal-receptors of avian epithelial cells. Amino acid substitutions were detected at the site within the receptor-binding domain as compared to the A/H9N2 influenza virus isolates obtained in Russia in 2018. The primary structure of the A/chicken/Chelyabinsk/30/2019 H9N2 isolate demonstrates a very high level of genetic similarity to the influenza virus isolate A/chicken/Israel/215/2007 H9N2 used as a vaccine strain.

Key words: avian influenza, H9N2, genetic analysis, amino acid substitutions.

INTRODUCTION

Avian influenza virus (AIV) belongs to the family *Orthomyxoviridae* that includes seven genera according to International Committee on Taxonomy of Viruses (ICTV) classification, Master Species List 2018b.v2 Release: *Alphainfluenzavirus* (A), *Betainfluenzavirus* (B), *Gammainfluenzavirus* (C), *Deltainfluenzavirus* (D), *Quaranjavirus*, *Thogovirus*, *Isavirus*. Type A influenza virus is classified into 16 hemagglutinin subtypes and 9 neuraminidase subtypes based on the structure of surface glycoproteins [4, 11].

Influenza viruses isolated from birds can be divided into two broad groups based on their virulence. Highly virulent viruses that cause acute generalized disease with up to 100% herd mortality are H5 and H7 subtypes. Viruses of other subtypes, as a rule, cause a mild, predominantly respiratory or asymptomatic disease and are classified as low virulent [1]. However, in case of inappropriate keeping conditions and presence of associated diseases, a clinical disease may manifest itself resulting in decreased productivity, culling of sick birds in the herd, and increased mortality of young chicks.

H9N2 subtype viruses are widespread in China, Southeast Asia, Pakistan, South Korea, Iran, Israel, India, and Africa in both wild birds and poultry [2, 3, 5, 7–9, 12, 14]. According to the published data, H9 subtype viruses form

several genetic groups: G1, Y280, and Y439 [1, 8, 10, 13]. G1 influenza virus isolates are endemic in the Middle East and Central Asia [8, 12]. In the Russian Federation, H9N2 subtype avian influenza was detected in the Amur Oblast in 2012, in the Primorsky Krai (the Far East) in 2018, in the Chelyabinsk Oblast in 2019. H9N2 subtype avian influenza viruses (AIVs) have recently become widespread among poultry and pose a real threat to the global poultry industry and potential danger to humans [1].

According to the current literature data [1, 2, 6] study of the genetic properties of A/chicken/Chelyabinsk/30/2019 H9N2 isolated in 2019 in the Chelyabinsk Oblast is of considerable interest and is the aim of this paper.

MATERIALS AND METHODS

Virus isolation was performed in 10-day-old specific pathogen-free chicken embryos (SPF CE). A 10–20% suspension was prepared in a phosphate-buffered solution (pH 7.2–7.4) from biological material and inoculated into the allantoic cavity of chicken embryos at 0.2 cm^3. Extra-embryonic fluid (EEF) was collected for testing from embryos that died after ≥ 24 hours of incubation.

RNA isolation. Total RNA isolation was performed using the RIBO-sorb kit (Cat. No. K2-1-Et-100) according to the manufacturer's instructions.

Real-time reverse transcription polymerase chain reaction (qRT-PCR). One-step qRT-PCR was performed using the OneStep RT-PCR Kit (Qiagen, Cat. No. 210212), 25 mM of $MgCl_2$ solution (Promega, Cat. No. M8296 kit) and H9 M and H gene primer sets. A mixture (25 µl) was formulated containing 9 µl of deionized water, 5 µl of 5× RT-PCR buffer, 1.25 µl of 25 mM $MgCl_2$ solution, 1 µl of 10 mM dNTP solution, 1 µl of a forward and reverse primer solution with a concentration of 10 pmol/µl, 0.75 µl of a fluorescent probe solution with a concentration of 10 pmol/µl, 1 µl reverse transcriptase and polymerase enzyme mixture, 5 µl of a total RNA solution. Reverse transcription was carried out at 50 °C for 30 min. The amplification was carried out based on the following temperature-time parameters: at 95 °C for 10 min (polymerase activation), then 40 cycles consisting of three steps each were run: at 95 °C for 10 seconds, at 50 °C for 35 seconds, at 72 °C for 10 seconds.

Reverse transcription polymerase chain reaction (RT-PCR). Classical one-step RT-PCR was performed using OneStep RT-PCR Kit (Qiagen, Cat. No. 210212), 25 mM $MgCl_2$ solution (Promega, Cat. No. M8296 kit) and H9 H gene primer set. 25 µl mixture was formulated and contained 9.75 µl deionized water, 5 µl 5× RT-PCR buffer, 1.25 µl of 25 mM $MgCl_2$ solution, 1 µl of 10 mM dNTP solution, 1 µl of a forward and reverse primer solution at a concentration of 10 pmol/µl, 1 µl of a reverse transcriptase and polymerase enzyme mixture, 5 µl of total RNA solution. Reverse transcription was carried out at 50 °C for 30 min. The amplification was carried out based on the following temperature-time parameters: at 95 °C for 10 min (polymerase activation), then 40 cycles consisting of three steps each were run: at 95 °C for 50 seconds, at 55 °C for 50 seconds, at 72 °C for 60 seconds.

Identification of the isolated virus. The virus was identified based on HA and NA with the hemagglutination inhibition test (HI test) using reference H1–H16 AIV sera (IZISVe, Italy) and neuraminidase activity inhibition test (NAI assay) using reference N1–N9 AIV sera (IZISVe, Italy). The tests were carried out based on the OIE recommendations and generally accepted methods [4, 15, 16].

Sequencing. The nucleotide sequences of the HA gene fragment were determined using ABI Prism 3100 automated sequencer and the BigDye Terminator Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's instructions. Full genome sequencing was performed using a MySeq genetic analyzer (Illumina) according to the manufacturer's instructions. Nextera XT, Nextera XT Index Kit (Illumina) commercial kits were used for library preparation.

Nucleotide sequences. Nucleotide sequences of H9 subtype AIV isolates and strains contained in GenBank database of the NCBI electronic resource (www.ncbi.nlm.nih.gov/nucleotide/) and on the EpiFlu platform were used in the present study (https://www.gisaid.org/).

The analysis of nucleotide sequences and corresponding amino acid sequences was performed using the BioEdit 7.0.5.3 version. Sequence alignment was performed using the ClustalW multiple alignment program. The phylogenetic tree was constructed using the NJ algorithm in MEGA software package version 6.06.

RESULTS AND DISCUSSION

In February 2019 20 samples of internal organs received from the poultry establishment in the Chelyabinsk Oblast were tested in the Reference Laboratory for Avian Viral Diseases (FGBI "ARRIAH") within state epidemic monitoring program. Suspended pathological material samples were inoculated to 10-day-old SPF chicken embryos. No mortality was observed during 72 hours of incubation. Hemagglutinating activity was observed in 14 extra-embryonic fluid samples collected from infected SPF chicken embryos. No virus was isolated from the other six samples of biological material during two consecutive passages in SPF chicken embryos.

The HI test using H1–H16 specific sera and NAI assay using N1–N8 reference sera showed that the isolated pathogen belongs to the H9N2 subtype.

AIV type A genetic material was detected and H9 subtype was identified in the samples by real-time RT-PCR. No H5, H7 subtype AIVs were identified in tested samples. HA gene fragment sequencing confirmed relationship of the isolates to the H9 subtype (presence of RSSR/GLF cleavage site).

A/chicken/Chelyabinsk/30/2019 H9N2 isolate showing the highest hemagglutinating activity was selected for further study (HA titer of 1:256).

The analysis of the HA gene nucleotide sequence of the isolated A/chicken/Chelyabinsk/30/2019 H9N2 showed a relationship with a group of isolates previously identified in Israel. The nucleotide sequence fragment of the hemagglutinin gene was used for phylogenetic analysis (1–1,539 bp open reading frame (ORF)). The analysis showed that the isolated A/chicken/Chelyabinsk/30/2019 virus belongs to the G1 genetic group (Fig. 1).

Further virus characterization was based on previous classification of H9N2 viruses isolated in Israel [8]. The analysis showed that the A/chicken/Chelyabinsk/30/2019 H9N2 virus belongs to a large group containing H9N2 clade IV virus isolates. This clade includes viruses isolated in Israel, Jordan and Lebanon in 2006–2012 [12]. The A/chicken/Israel/215/2007 virus was taken as basic for comparative study as it was previously the most characterized one and used as a vaccine strain [8].

Taking into account that low-virulent influenza viruses can circulate in nature among wild birds for a long time

without causing epidemics, it is important to carry out full-genome sequencing of these isolates. The main goal of the full-genome sequencing of influenza viruses is deep analysis of the viral genome taking into account various parameters, including checking for a possible antigenic shift in the newly isolated virus, the presence of significant amino acid substitutions in the structure of the viral proteins, and the establishment of probable parental forms and geographical origin of the virus.

The available nucleotide sequences with ORFs of the virus's closest phylogenetic neighbors recovered in Israel (A/chicken/Israel/215/2007), Russia (A/chicken/Siberia/03/2018, A/chicken/Amur_Russia/17/2018), Egypt (A/chicken/Egypt/D7099/2013), Lebanon (A/quail/Lebanon/273/2010) were used for comparative analysis. The isolated A/chicken/Chelyabinsk/30/2019 virus was found to have a very high level of genetic similarity to the A/chicken/Israel/215/2007 H9N2 isolate (Table).

The high level of genetic similarity in all genome segments of A/chicken/Siberia/03/2018, A/chicken/Amur_Russia/17/2018, A/chicken/Chelyabinsk/30/2019 isolates indicates the absence of reassortment with other influenza virus subtypes previously identified in the Russian Federation.

The analysis of the HA protein amino acid sequence of the A/chicken/Chelyabinsk/30/2019 H9N2 isolate showed presence of $G_{225}LIG_{228}$ amino acids (according to H3 subtype numbering) at the receptor-binding site of the viral protein (Fig. 2).

The amino acid composition of $G_{225}LIG_{228}$ suggests affinity for the ACA2-3Gal avian receptors and absence of interaction with ACA2-6Gal mammalian receptors [17]. Thus, birds should be considered the main carriers of the A/chicken/Chelyabinsk/30/2019 H9N2 virus. However, there are studies showing that Q226L substitution facilitates interaction of influenza virus with human respiratory tract receptors [18].

Apart from that it should be noted that the heterogeneity of the virus hemagglutinin receptors has already been formed among the AIV H9N2 subtype isolates recovered in Russia. Analysis of the amino acid sequence of the HA protein of A/chicken/Chelyabinsk/30/2019 H9N2 isolate revealed amino acid substitutions at the site of the receptor-binding domain at positions 160, 181, 226, 227 (according to H3 numbering) (Fig. 2).

Analysis of the predicted amino acid sequence showed a high similarity (up to 100%) between the A/chicken/Chelyabinsk/30/2019 viruses and the previously described A/chicken/Israel/215/2007 as regards other viral genes. An extremely small number of significant nucleotide substitutions and the absence of differences in key markers between A/chicken/Chelyabinsk/30/2019 and A/chicken/Israel/215/2007 isolates suggest that the biological properties of the compared viruses are similar.

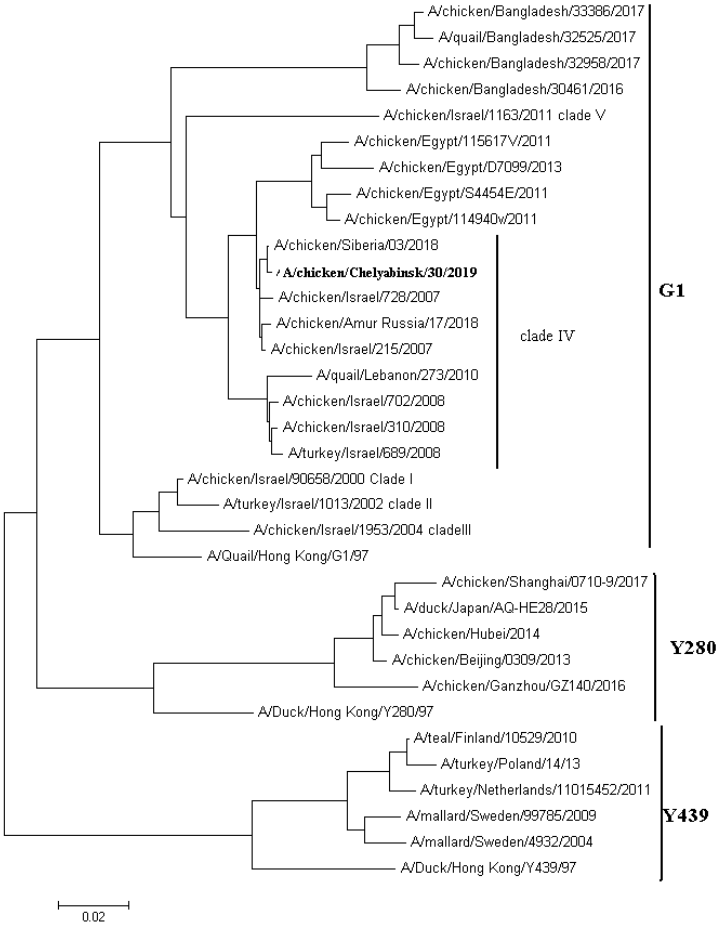

Fig 1. Phylogenetic tree constructed using the NJ method based on the nucleotide sequence of the AIV H9 HA gene fragment (1–1,539 bp ORF)

The identified high genetic similarity between A/chicken/Chelyabinsk/30/2019 virus and the previously identified A/chicken/Siberia/03/2018 and A/chicken/Amur_Russia/17/2018 isolates suggests that the virus was introduced into the territory of the Russian Federation from a small geographical area. For your reference see available information on H9N2 influenza virus isolates recovered in the People's Republic of Bangladesh [7]. According to the data presented, genetic heterogeneity among the G1 viruses from Bangladesh is much greater. Thus, the HA gene

Table
Level of A/chicken/Chelyabinsk/30/2019 H9N2 genetic similarity for different genes

H9N2 AIV isolate	Compared gene, %							
	HA	NA	PB1	PB2	M	NP	PA	NS
A/chicken/Israel/215/2007	99.4	99.4	99.6	99.9	99.6	99.9	99.8	99.6
A/chicken/Siberia/03/2018	99.8	99.7	99.8	99.8	99.6	99.9	99.8	99.7
A/chicken/Amur_Russia/17/2018	99.3	99.5	99.7	99.7	99.6	99.7	99.8	99.5
A/chicken/Egypt/D7099/2013	96.2	95.6	96.2	93.6	98.4	95.6	94.5	96.2
A/quail/Lebanon/273/2010	96.5	97.8	90.9	88.2	91.4	90.5	92.1	83.0

Fig. 2. The predicted amino acid sequence site of the virus hemagglutinin receptor-binding domain

difference between the isolates recovered in 2016 and 2017 reaches 3%. The geographic remoteness of the areas where A/chicken/Siberia/03/2018 (Novosibirsk Oblast), A/chicken/Amur_Russia/17/2018 (Amur Oblast) and A/chicken/Chelyabinsk/30/2019 (Chelyabinsk Oblast) were isolated also indicates the only source of the G1 genetic group virus introduction into the Russian Federation. Unfortunately, it is extremely difficult to establish the source of introduction as H9N2 viruses are low pathogenic for wild birds and poultry and long-term asymptomatic carriage may occur.

CONCLUSION

The study showed that the A/chicken/Chelyabinsk/30/2019 virus belongs to the G1 genetic group of H9 subtype. Full-genome sequencing suggested lack of reassortment of genomic segments with regard to the G1 genetic group H9N2 subtype viruses previously identified in the Russian Federation. A high level of similarity of the detected virus with the A/chicken/Israel/215/2007 virus was established. An analysis of the virus molecular markers indicates the adaptation of the A/chicken/Chelyabinsk/30/2019 virus to the bird population.

Conflict of interest. The authors declare no conflict of interest.

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